CHANGES IN PROTEIN EXPRESSION IN MATURING EQUINE TESTIS: A QUANTITATIVE DIGE ANALYSIS

A Senior Scholars Thesis

by

PILAR ROPER-FOO

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2009

Major: Genetics

CHANGES IN PROTEIN EXPRESSION IN MATURING EQUINE TESTIS: A QUANTITATIVE DIGE ANALYSIS

A Senior Scholars Thesis

by

PILAR ROPER-FOO

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by:

Research Advisor: Lawrence J. Dangott
Associate Dean for Undergraduate Research: Robert C. Webb

April 2009

Major: Genetics

ABSTRACT

Changes in Protein Expression in Maturing Equine Testis: A Quantitative DIGE Analysis. (April 2009)

Pilar Roper-Foo
Department of Biochemistry & Biophysics
Texas A&M University

Research Advisor: Dr. Lawrence J. Dangott
Department of Biochemistry & Biophysics

Gross observation of testicular parenchyma of prepubertal horses reveals both light and dark regions. The light regions are associated with localized initiation and regulation of spermatogenesis. It is hypothesized that different proteins and protein expression levels in the light regions are factors contributing to successful sperm production. Differential Gel Electrophoresis (DIGE) and DeCyder Image analysis of testicular extracts from prepubertal stallions has identified forty proteins that may be involved in the regulation of the onset of spermatogenesis. Preliminary LC/MS/MS mass spectrometric analysis of six protein spots has been analyzed, but no protein identifications have yet been made. The results of this preliminary research are inconclusive with respect to protein identification, but the developments of protocols for procurement, protein extraction of light and dark tissue and DIGE analysis have been successful.

DEDICATION

I would like to dedicate this thesis to my parents, Edward and Nancy, for always encouraging me to succeed.

ACKNOWLEDGMENTS

I would like to acknowledge Dr. Larry Johnson for his financial support and assistance with the sample collection process. I would also like to thank Drs. Martin and Griffin for their veterinarian expertise. To Sabrina Schmidtke, thanks for all the help with laboratory work as well as with the mass spectrometric analysis. Lastly, I would like to thank Dr. Dangott, not only for his financial support and research guidance, but more importantly for his unyielding support and encouragement.

NOMENCLATURE

SDS PAGE	**S**odium **D**odecyl**s**ulfate **P**oly**A**crylamide **G**el **E**lectrophoresis
DIGE	**Di**fference **G**el **E**lectrophoresis
CHAPS	3-[3-(**Ch**ol**a**midopropyl)dimethylammonio]-1-**p**roane**s**ulfonate
TRIS	Trisma Base
IPG	**I**mmobilized **p**H **G**radient
PBS	**P**hosphate **B**uffered **S**aline
LC/MS/MS	**L**iquid **C**hromatography/**M**ass **S**pectrometry/**M**ass **S**pectrometry
N_2	Liquid **N**itrogen
DTT	**D**i**t**hio**t**hreitol
IAA	**I**odo**a**cet**a**mide
DIA	**D**ifference **I**n-gel **A**nalysis
BVA	**B**iological **V**ariation **A**nalysis
UTSB	**U**rea/**T**hiourea **S**ample **B**uffer
IgG	**I**mmuno**g**lobin **G**
L-buffer	**L**abeling buffer

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Spermatogenesis occurs uniformly in many mammalian species [1], resulting in the corresponding uniform development and coloration of the parenchymal tissue. In contrast, spermatogenesis in horses is uniquely localized to regions of the testis, with distinct coloration between mature and immature tissue. A cross section of testis from young horses shows the lightly colored parenchyma associated with maturing spermatogenic cells in the center, and darker, immature tissue surrounding the periphery [2].

Clemmons et al. [3] have shown that gross differences in coloration within the parenchyma of the same testis correspond to quantitative differences in cell populations in that testis. The dark region is characterized by large numbers of Leydig cells, macrophages and small seminiferous tubules that are not producing sperm. In contrast, the light tissue is composed of fewer Leydig cells and macrophages as the seminiferous tubules (containing non-pigmented cells) increase in size to occupy a larger proportion of the parenchyma. A pattern emerges with lighter, central regions that are spermatogenically active and a darker periphery remaining relatively inactive.

This thesis follows the style of Biology of Reproduction.

Previous reports using microarray illustrated that differentiated expression of mRNAs between light and dark [4]. Of the 9132 human genes studied, microarray with equine cDNAs revealed that expression of at least 88 equine genes is different between light and dark tissue.

Proteins are directly responsible for the onset of initiation of spermatogenesis and we would like to study the proteomic functions involved spermatogenesis, particularly factors that function in the onset of imitation. Additionally, investigation into protein expression level changes of light and dark tissue could possibly link specific proteins to their mRNA counterparts [4].

The goal of the present work is to begin to identify proteins that may be involved in the onset and propagation of spermatogenesis by utilizing Difference Gel Electrophoresis (DIGE) and mass spectrometry. Similar experiments using the DIGE technique have already been used to identify proteins preferentially expressed at each stage of rat spermatogenesis [5]. Published results found 265 protein spots displaying differential expression ultimately leading to 123 protein identities. By identifying proteins that are differentially expressed in dark and light parenchyma of the developing equine testis, we hope to establish the horse as an animal model to study localized sperm production and biological mechanisms that control spermatogenesis.

CHAPTER II

METHODS

Materials and methods

Specimens

Testes from pre-pubertal horses were obtained from 1-2 year old stallions raised at the Texas State Prison Farm (Wynne Prison Farm) in Huntsville, Texas and from the Horse Center at Texas A&M University in College Station, Texas. The testicles were catalogued using the stallions' ID numbers, segregated according to position (left or right) and weighed. The testicles were then sectioned and dissected into light or dark regions [2], rinsed in ice-cold PBS and immediately frozen with liquid nitrogen (N_2).

Protein extraction

A variety of treatments were tested before settling on the final protocol presented here. Approximately 125 milligrams of tissue was pulverized under liquid N_2, and dissolved in 1 milliliter of 10mM Tris, pH 7.5, 4% CHAPS, that contained a protease inhibitor cocktail (Complete Cocktail Tablets, Roche Diagnostics, Mannheim, Germany) for 1 hour on ice. This was followed immediately by the addition of a Ribonuclease A/Deoxyribonuclease I cocktail, (Worthington Biochemical Corporation, Lakewood, NJ) for an additional 15 minutes on ice. The materials were submitted to centrifugation at 13000 x gravity for 5 minutes at 4^{o} C. Protein was estimated by the Bradford assay [6] using albumin as a standard. The extracted materials were evaluated by SDS PAGE [5].

Albumin and IgG depletion

Equine IgG and albumin were depleted using immunoaffinity chromatography (Seppro Beads, GenWay Biotech, San Diego, CA) on an ÄKTA Explorer 10 chromatography system. Anti-Human IgG and Anti-Human Albumin affinity columns (2 ml volume) were coupled in tandem. Sample loading on the column was determined by estimating the amount of albumin in the sample using SDS PAGE and scanning densitometry using ImageQuant 5.1 software (GE Healthcare) and then assuming 50% binding capacity due to the interspecies nature of the experiment. The relative amount of albumin in the samples was calculated as to be approximately 25% of total protein.

Approximately 750 micrograms of extracted testis proteins were injected onto the tandem columns and a step gradient was developed according to the manufacturer's recommendations. Proteins collected in the flow-through fractions from this "piggy back" method were analyzed by SDS PAGE and the albumin and IgG-free fractions were pooled for subsequent analysis.

DIGE labeling and electrophoresis

Pooled flow through fractions of IgG and albumin depleted samples were spin-concentrated using a Vivaspin 15 R-10,000 MW cut off (Sartorius Biolab, Goettingen, Germany) in a Sorvall RC-3B centrifuge and precipitated using a Chloroform/Methanol protocol developed by Wessel et al. [7]. After solubilization in L- buffer (7M Urea/2M Thiourea, 4% CHAPS, 30mM Tris, pH 8.5), the proteins were differentially labeled at a

ratio of 200 pmol dye/50 ugm protein with spectrally resolvable fluorescent CyDyes (GE Healthcare). Isoelectric focusing was performed on an IPGPhor focusing unit (GE Healthcare) with IPG Drystrips (GE Healthcare, pH 4-7, 13 cm) for a total of 35,000 Volt-hours. According to Unlu et al., a pooled internal standard composed of an equal amount of protein from all samples was labeled with Cy2 and included as a normalization standard for subsequent DeCyder analysis [8]. The focused proteins were subsequently reduced with DTT and alkylated with IAA and run on a 12% acrylamide 2D SDS PAGE slab gel. The gels were imaged at of 532 nanometers (Cy 3), 633 nanometers (Cy 5), and 488 nanometers (Cy 2) on a Typhoon Trio Imager (GE Healthcare). Images were analyzed and cropped using Image Quant 5.1 prior to multi-channel analysis using DeCyder Software (version 6.5).

DeCyder image analysis

DeCyder image analysis software (Version 6.5, GE Healthcare) was used to detect, match and compare migration of individual proteins for all protein spots in the multiple fluorescent images. Proteins were detected using the Differential In-gel Analysis (DIA) module and an exclusion filter (Slope > 1.85, Area > 272, Volume > 230000, Peak Height between 1000-100000) was set to remove all non-protein spots from the protein list. The Biological Variation Analysis (BVA) module was used to match and compare protein spots between gels, and a statistical filter was applied (Protein is present in both gels, Student's T-Test < 0.1, Average ratio < -1.5 but >1.5, volume >1.0e5 but < 1.0e8) to detect proteins with gel-to-gel differences. Forty proteins met these criteria and were

designated as “pick” proteins that were removed from the gel for further analysis. A representative gel was post-stained with Deep Purple (GE Healthcare), imaged with the Typhoon and used for spot picking.

Spot picking and digestion

Gel plugs containing proteins of interest were cored from the gel with an Ettan Picker robot (GE Healthcare). Picked gel plugs were transferred in a 96-well plate and placed in an Ettan Digester (GE Healthcare). Automated digestion with trypsin was performed using a program that followed the method of Shevchenko et. al. [9]. Extracted peptides were stored in -80^{o}C while awaiting mass spectrometric analysis.

LC/MS/MS analysis and database search

Nanospray LC/MS/MS was performed on an LCQ DecaXP 3D Ion Trap Mass Spectrometer (ThermoFinnigan, San Jose, CA). Extracted peptides were concentrated by vacuum centrifugation to dryness and then dissolved in 12 microliters of 98% solution A (0.1% Formic Acid in water) and 2% solution B (0.1% Formic Acid in acetonitrile) for 30 minutes at room temperature. The samples were injected onto a hand-packed, 6 cm PicoFrit column (New Objective, Woburn, MA) containing Magic C18AQ resin (5 micron, 200 Å pore size, Michrom, Auburn, CA). Protein sequences obtained from electrospray were used to search the National Center for Biotechnology Information non repetitive (NCBI-nr) database (July 29-30, 2008) using an in-house copy of the automated search engine MASCOT (2008, Version 2.2.1.0) after extracting the data with

the Distiller module (Matrix Science). Search parameters were set to account for any modifications made to the proteins during extraction and digestion. The parameters were: enzyme used was trypsin, search Mammalia database, allow 1+, 2+, 3+ ion charges, allow 1 missed cleavage, carbamidomethylated cysteine and oxidation of methionine.

CHAPTER III

RESULTS AND DISCUSSION

An initial primary concern of this project was that the presence of large quantities of blood proteins would obscure the presence of more minor testicular proteins of interest, thus our first interest was the removal of blood proteins (albumin and IgG) from the tissue extracts. Although attempts to reduce the quantity of contaminating blood proteins were made at the time of collection, SDS PAGE consistently detected large quantities of blood proteins (estimated at >30% albumin and IgG) in the samples. Figure 1 is an SDS PAGE comparison of the different treatments used to reduce the amount of albumin in the samples during collection. Two treatments to reduce/remove blood proteins were tested; intravenous perfusion with saline and gross washing. As shown, the difference in albumin in the non-perfused and perfused samples (as based on total protein staining) in lanes 2, 4 and 5 respectively, was minimal, suggesting only partial removal of the blood proteins. In contrast, gross washing of the tissues in PBS removed the albumin to a greater extent than other methods tested (lanes 3 and 6). Since the washed samples had the smallest amount of albumin, and washing the tissue is much easier than perfusion, this method was used in all subsequent treatment of tissue.

Two protein extraction methods were tested. The first method tested was direct extraction of the proteins in L-buffer. This resulted in a viscous sample that was highly contaminated with chromosomal DNA and blood proteins, both of which would interfere

in subsequent fluorescent labeling and DIGE analysis. The second method, extracted with TRIS/CHAPS, proved to yield better separation, higher resolution and less protein degradation than the first method due mostly to the incorporation of a protease inhibitor (PI) and a nuclease cocktail. The UTSB/CHAPS method, which did not utilize PI and nuclease, resulted in a more viscous supernatant. In addition, the UTSB/CHAPS method was incompatible with subsequent immunoaffinity depletion. Figure 2 shows the results of the two protein extraction methods evaluated in this project. Regions A, B, and C of Figure 2 show reduced protein degradation and higher resolution yielded by the Tris/CHAPS method (lanes 4, 5). This method was therefore used in all subsequent extractions for this project.

As shown above, washing the dissected tissue with PBS only removed a minimal amount of albumin from the sample. In order to remove more albumin, immunoaffinity chromatography was employed. In preliminary experiments, 1 milligram of protein extracted by the Tris/CHAPS method was passed through an anti-albumin column. Albumin-depleted fractions were subsequently collected, pooled together and concentrated down to approximately 300 microliters before being run through the anti-IgG column. Figure 3 illustrates which flow-through fractions containing depleted albumin were selected to be run on the anti-IgG column. Successful separate depletion of both albumin and IgG (Figures 4 and 5) led to the coupling of the two columns in tandem.

Light and dark tissue from the same testis was albumin and IgG depleted in triplicate in order to obtain enough protein material for the DIGE experiment. Fractions from the tandem immunoaffinity columns were analyzed by SDS PAGE to determine which fractions contained significant amounts of protein to begin DIGE.

DIGE and DeCyder Image Analysis resulted in the detection of 882 proteins in the testis sample. Statistical limits set in the BVA module identified 40 proteins of statistical significance. Figure 6 illustrates a representative DIGE gel. The red circles indicate which spots were picked from the gel for digestion and LC/MS/MS analysis.

Table 1 illustrates the pick list. The master number represents the position of the protein with respect to the gel. The T-test assesses the statistical difference between the light and dark populations. Average ratio indicates the change in protein expression levels. Twenty-one of the 40 proteins in the dark tissue had negative average ratios, indicating a decrease in protein expression relative to the light tissue. Nineteen proteins in the dark tissue had positive average ratios indicating increased protein expression relative to the light tissue. Protein spot volume measures the pixel count of each spot; the higher the volume, the more intense the spot and, presumably the more protein present. The list is sorted by spot volume. Based upon the spot volume, digests of the six most abundant proteins were selected for LC/MS/MS analysis.

Results from the Mascot search were inconclusive. Table 2 above indicates that no protein identification was made from any of the six proteins analyzed in these initial experiments. This is presumably due to the inadequate amount of protein in the spots that may be correctable by decreasing sample loss during processing. Sample loss may have occurred at several stages of the experiment: Chloroform/methanol precipitation step, during resolubilization in L-buffer (separate resolubilization experiments indicate a loss of approximately 30% of protein during resolubilization; Dangott, unpublished results), and during isoelectric focusing. (There are a number of unknown causes of sample loss associated with 2D gel electrophoresis; Dangott, unpublished results.)

One of the primary goals of this project was to ask whether a relationship exists between the protein expression level changes in light and dark tissue and other biologically significant changes occurring in the testes during spermatogenesis. Ing et. al. [4] have reported changes in approximately 90 specific messenger RNAs in light and dark tissue based on microarray data. In those studies, dysferlin, down-regulated in ovarian cancer I and Golgi apparatus protein I were preferentially expressed in dark tissues while outer dense fiber of sperm tails and phoshpodiesterase 3B were more highly expressed in light tissues.

Our study attempted to identify what changes occur at the protein level and, although inconclusive due to technical problems, we have demonstrated that at least 40 proteins change expression level in the light tissue compared to the dark. It is our goal to identify

these proteins in order to correlate their expression to the changes reported in mRNA expression by Ing [4].

Interestingly, we have located several protein spots by DIGE that appear to correlate in pI and molecular weight with predicted gene products of several of the mRNAs detected by Ing et. al [4]. For example, phosphodiesterase 3B, Ste-20 related kinase Mad31, Sortilin and Angio-associated migratory cell protein were identified as mRNAs that increased in light tissue. Although the proteins we have identified have similar electrophoretic characteristics as those predicted, expression levels, based on DeCyder analysis do not correlate in all cases with changes in mRNA levels reported by Ing [4]. It is known, however, that mRNA levels do not always accurately predict changes in protein levels and that the changes we have identified may indeed have biological meaning beyond what is implied by the microarray data. Further study of the proteome is required including positive identification of the proteins highlighted in this study.

CHAPTER IV

CONCLUSION

Light and dark tissue extracts of equine testicular tissues were successfully analyzed using the DIGE system and resulted in the identification of at least 40 proteins that display altered expression levels in the two populations of spermatogenic tissue. During the course of the study, significant advances were made in the development of sample collection and protein extraction methods that provided the basis for improved protein labeling and resolution and will allow for further quantitative analysis of the equine testicular proteome during spermatogenesis.

REFERENCES

1. Courot M, Hochereau-de Reviers M-T, Ortavant R. Spermatogenesis. In: Johnson AD, Gomes WR, Van Demark NL, (eds.) The Testis. Vol. I. New York: Academic Press; 1970: 339-442.

2. Johnson L. Spermatogenesis. In: Cupps PT (ed.), Reproduction in Domestic Animals. 4th ed. New York: Academic Press; 1991: 173-219.

3. Clemmons AJ, Thompson DL Jr., Johnson L. Local initiation of spermatogenesis in the horse.
Biol Reprod 1995; 6:1258-1267.

4. Ing NH, Laughlin AM, Varner DD, Welsh TH Jr., Forrest DW, Blanchard TL, Johnson, L. Gene expression in the spermatogenically inactive "dark" and maturing "light" testicular tissues of the prepubertal colt. J Androl 2004; 25:535-544.

5. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-685.

6. Bradford, M.M. (1976). A rapid and sensitive method for the quantification of

microgram of protein utilizing the principle of protein-dye binding. Anal. Biochem 72:248-254.

7. Wessel D, Fugge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Analytical Biochemistry 1984; 138: 141-143.

8. Unlu M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 1997; 18: 2071-2077.

9. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nature Protocols 2006; 1: 2856-2860.

APPENDIX A

FIGURES

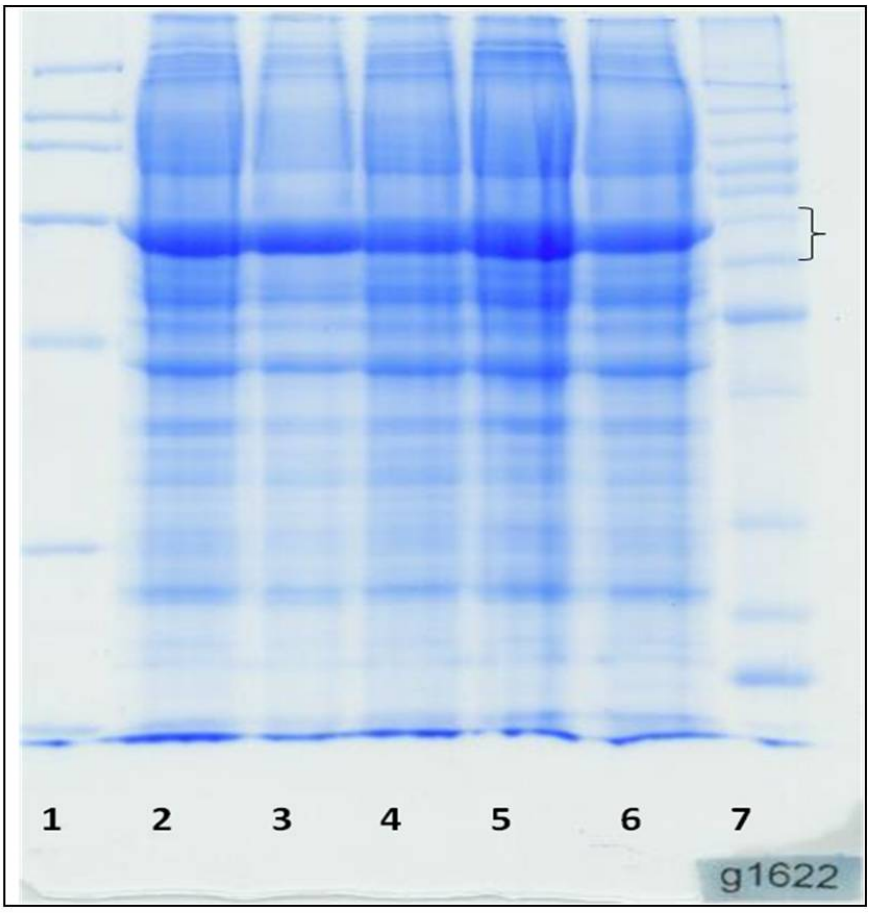

Figure 1. SDS PAGE comparison of perfused, non-perfused and washed samples. Lanes 1,7= MW Stds, 2= D2, Tris/CHAPS, dark (non-perfused), 3= D#82, Tris/CHAPS, dark (washed), 4= D#32, Tris/CHAPS, dark (perfused), 5= L2, Tris/CHAPS, light (non-perfused, 6= L#82, Tris/CHAPS, light (washed). Bracket indicates region of albumin migration.

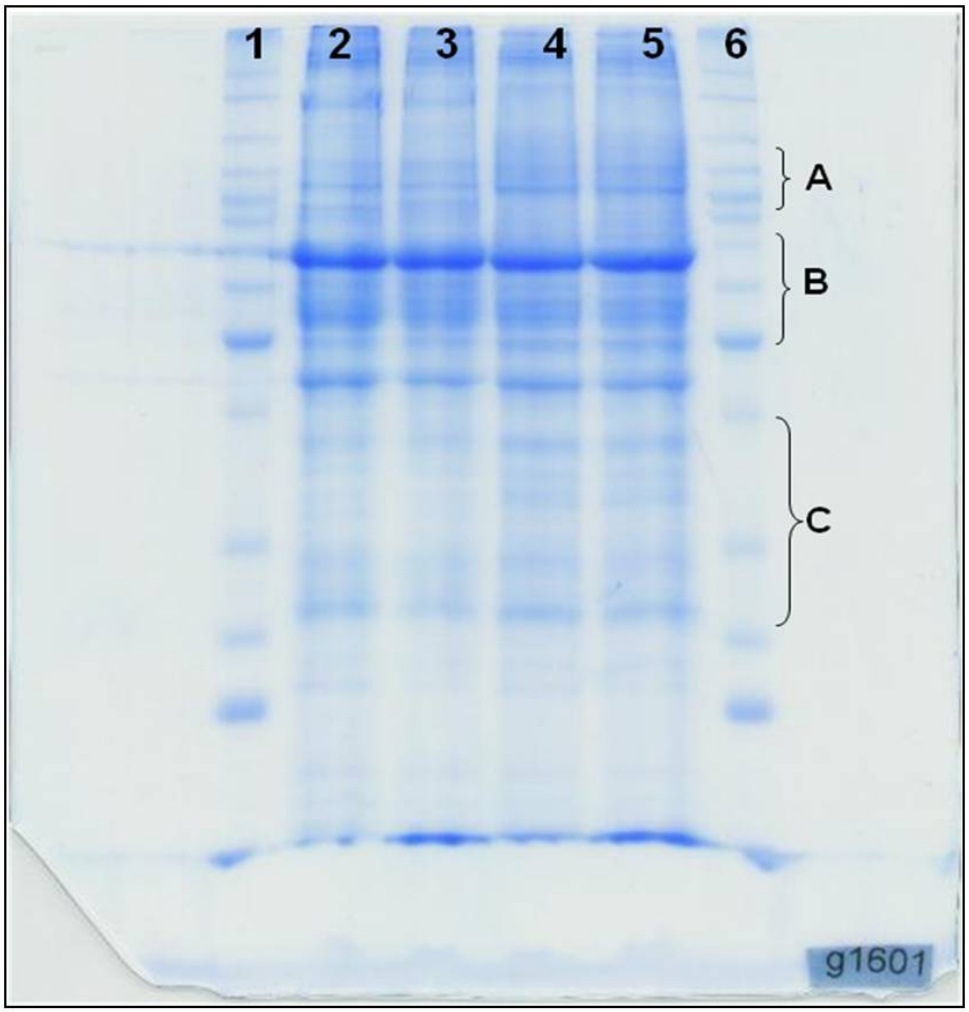

Figure 2. SDS PAGE comparison of two sample extraction methods. Lanes 1,6= MW Stds, 2= L1, UTSB/CHAPS, 3= D1, UTSB/CHAPS, 4= L2, Tris/CHAPS, 5= D2, Tris/CHAPS.

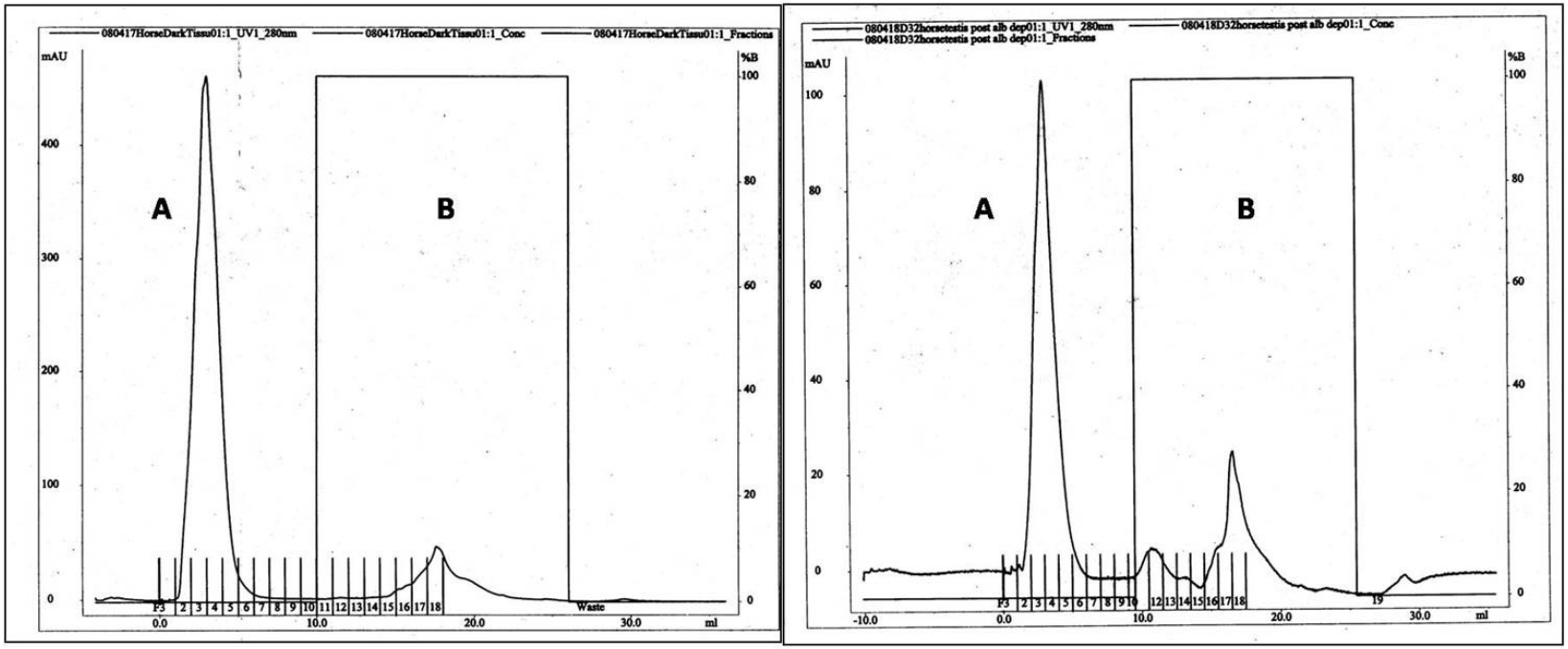

Figure 3. Depletion chromatograms: albumin and IgG
Left- Chromatogram of albumin depleted protein.
Right- Chromatogram of IgG depleted protein.
Region A represents flow-through fractions containing depleted sample to be analyzed.
Region B represents albumin or IgG being removed from the column to the waste.

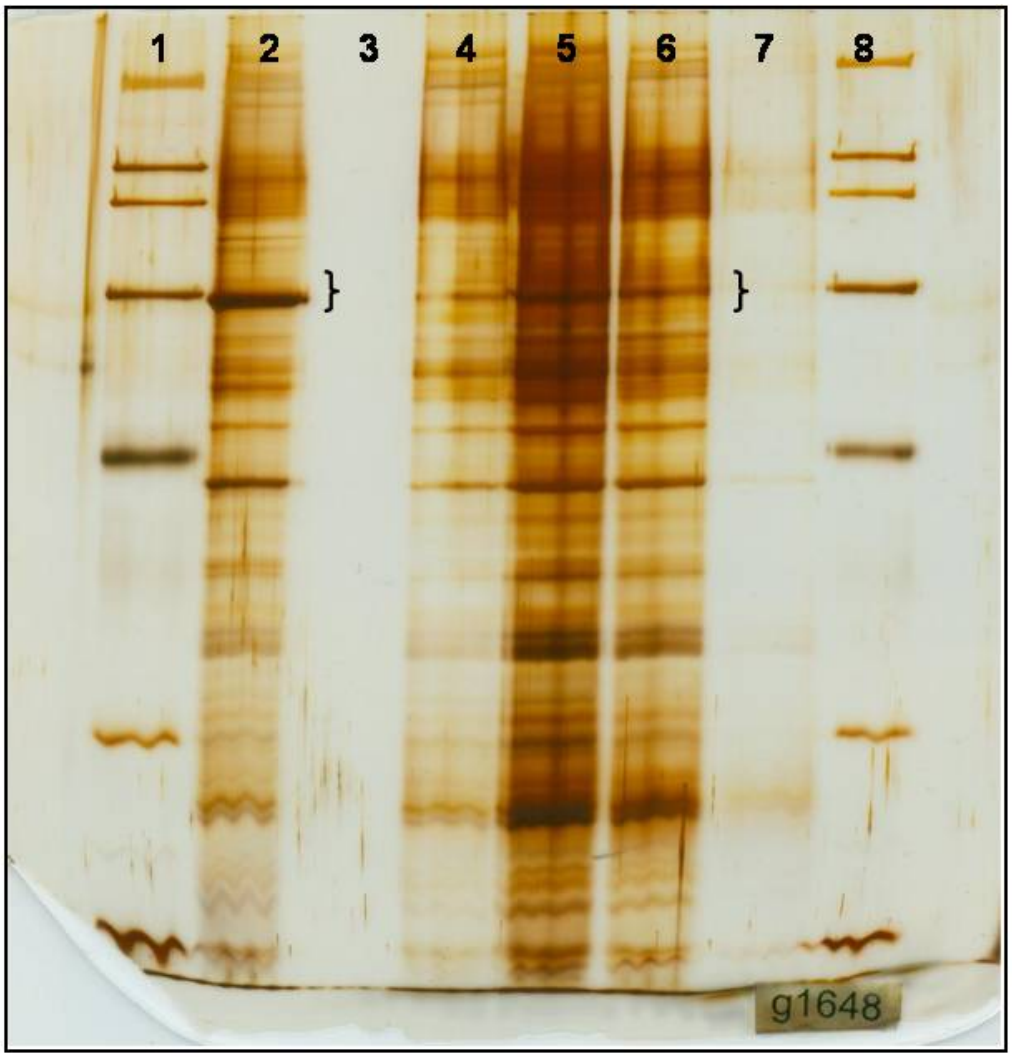

Figure 4. Albumin Depletion. Flow through fractions after Albumin depletion only. Lane: 1,8= MW Stds, 2=Stock D#32 (pre-albumin depletion), 3= fraction 2, 4=fraction 3, 5=fraction 4, 6=fraction 5, 7=fraction 6. Brackets indicate region of albumin migration. Note the greatly reduced amount of albumin in depleted fractions.

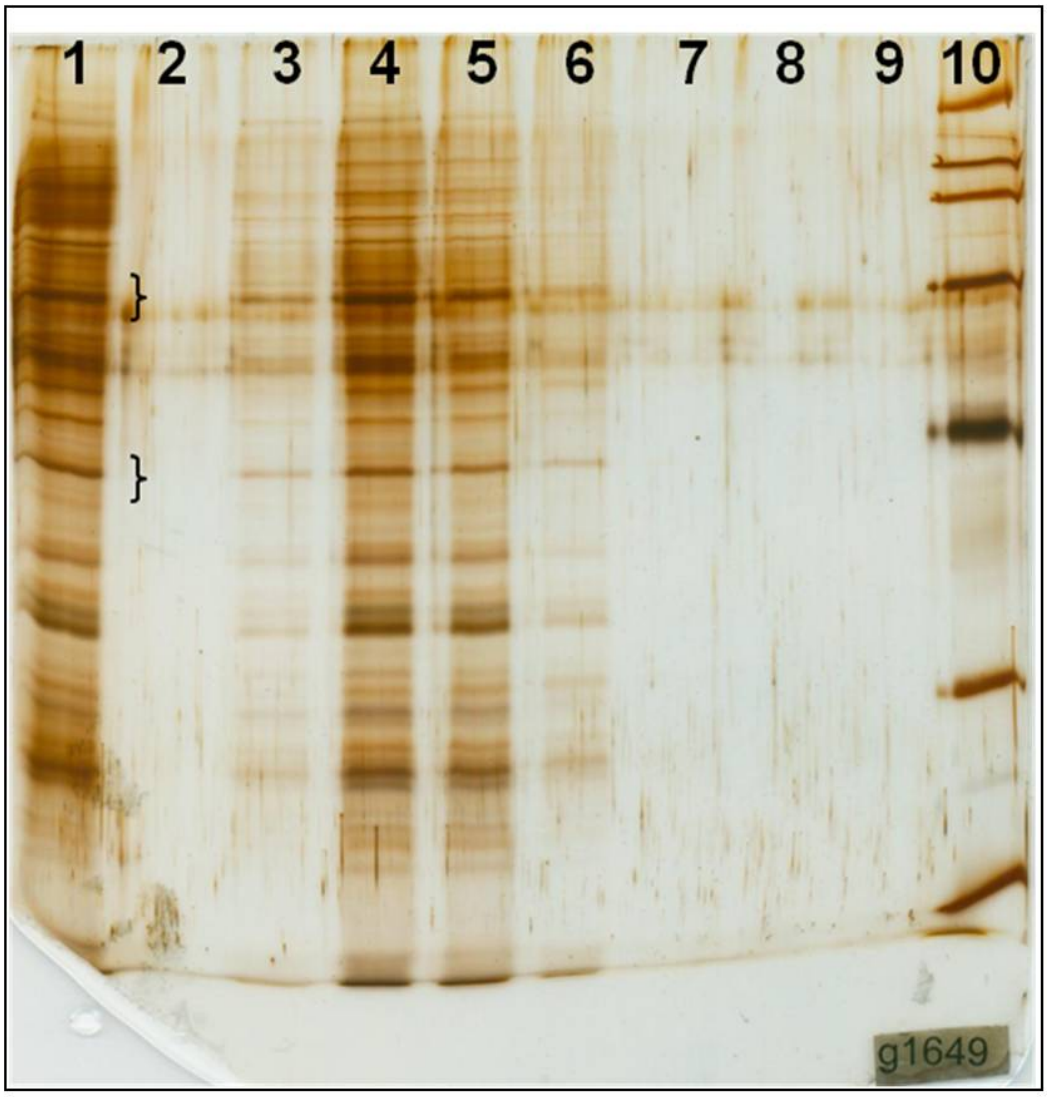

Figure 5. Flow-through fractions after albumin and IgG depletion. Lane 1= Stock D#32 (pre-albumin and IgG depletion), 2= fraction 2, 3= fraction 3, 4= fraction 4, 5= fraction 5, 6= fraction 6, 7= fraction 7, 8= fraction 8, 9= fraction 9, 10= MW Std. Brackets indicate regions of albumin and IgG migration, respectively.

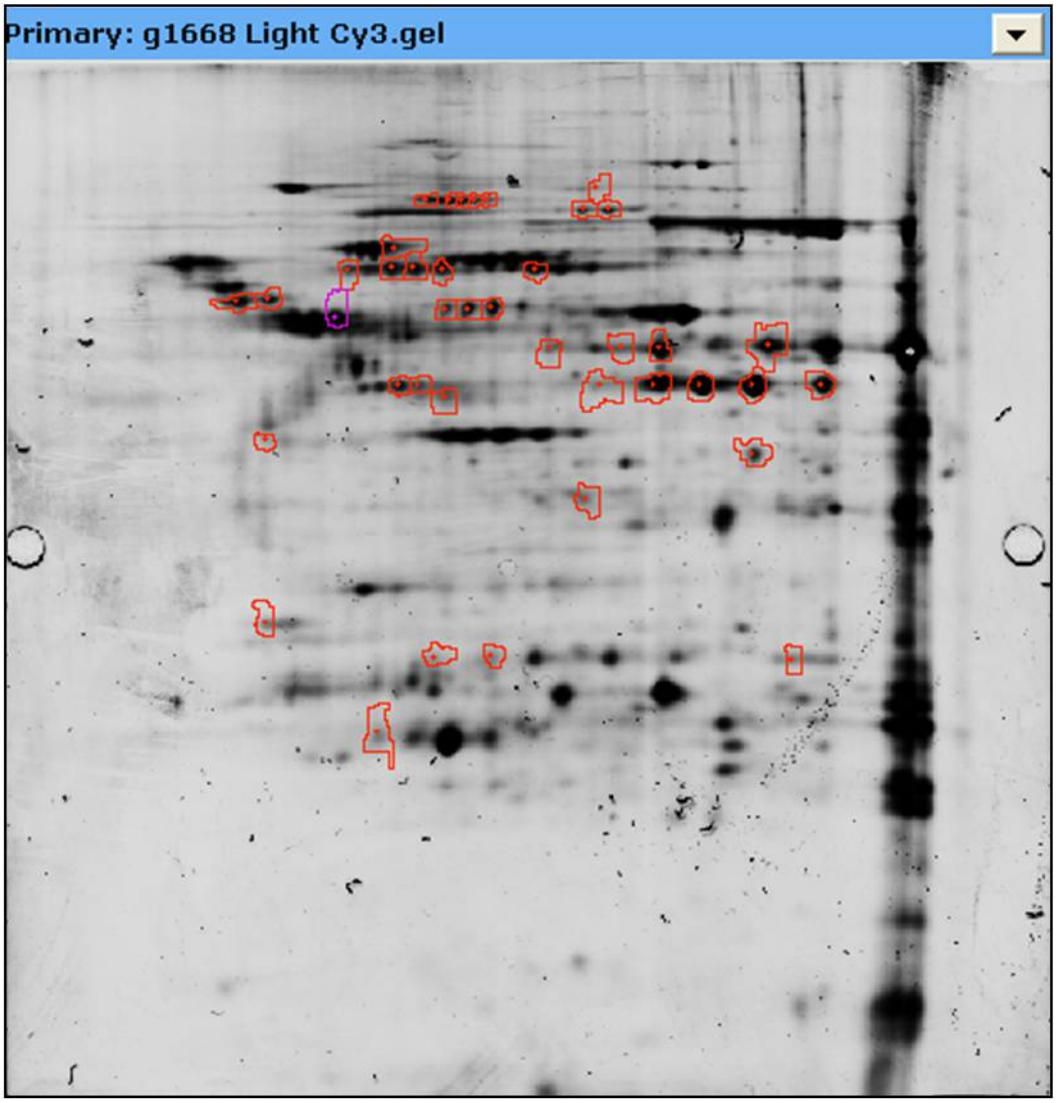

Figure 6. A completed DIGE gel: Cy 3 labeled. Circled areas represent the 40 proteins picked from the gel and digested with Trypsin.

APPENDIX B

TABLES

Table 1. DeCyder analysis: protein pick list generated in BVA. The table is arranged by spot volume. The first six were chosen for LC/MS/MS and searched in Mascot Distiller.

Protein #	Master No.	T-test value	Avg. Ratio	Spot Vol.
1	358	0.081	-3.2	35523726
2	231	0.081	1.49	25507854
3	373	0.081	-2.84	24835983
4	626	0.085	1.45	24199838
5	346	0.081	-4.22	20989208
6	165	0.081	1.39	16336759
7	167	0.081	1.36	16120900
8	170	0.081	1.32	14058932
9	370	0.081	-2.31	12939768
10	386	0.081	1.23	12771411
11	316	0.081	-2.12	12677700
12	180	0.081	1.18	9928108
13	288	0.085	-1.92	9230901
14	667	0.081	1.72	7225116
15	225	0.081	-2.04	7096686
16	350	0.081	-1.16	7059916
17	73	0.081	1.41	6558315
18	130	0.081	1.15	6166242
19	368	0.081	-1.51	5831471
20	456	0.081	-1.19	5760383
21	663	0.081	1.83	5477628
22	353	0.081	-1.1	5474022
23	662	0.081	2.07	5000616
24	222	0.081	-1.73	4957504
25	504	0.081	-1.52	4728005
26	161	0.081	1.42	4460243
27	304	0.081	-1.78	4194804
28	207	0.081	1.26	4021881
29	426	0.081	1.35	3583718
30	69	0.081	1.34	3523316
31	779	0.081	1.69	3021670
32	53	0.081	-2.65	2908610
33	221	0.081	-1.41	2331470
34	43	0.081	2.1	2275352
35	315	0.081	-1.84	2239252
36	57	0.073	-3.54	2237943
37	54	0.081	-3.35	2176467
38	56	0.073	-3.54	2097758
39	204	0.081	1.45	1757906
40	55	0.073	-3.28	1667417

Table 2. Select proteins analyzed by LC/MS/MS. Each of the six proteins below was searched using Mascot Distiller. All six proteins yielded no protein identification.

Spot Number	Master Spot Number	Avg. Ratio Change	Protein Identification
1	358	-3.20	No ID
2	231	1.49	No ID
3	373	-2.84	No ID
4	626	1.45	No ID
5	346	-4.22	No ID
6	165	1.39	No ID

CONTACT INFORMATION

Name:	Pilar Roper-Foo
Professional Address:	c/o Dr. Lawrence J. Dangott Department of Biochemistry/Biophysics MS 2128 Texas A&M University College Station, TX 77843
Email Address:	properfoo@neo.tamu.edu
Education:	B.S., Genetics, Texas A&M University, May 2009 Minor, Chemistry Undergraduate Research Scholar